AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VACCINES AND ANTIVIRAL AGENTS

Adjuvant-Dependent Enhancement of HIV Env-Specific Antibody Responses in Infant Rhesus Macaques

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ABSTRACT Toward the goal of developing an effective HIV vaccine that can be administered in infancy to protect against postnatal and lifelong sexual HIV transmission risks, the current pilot study was designed to compare the effect of novel adjuvants on the induction of HIV Env-specific antibody responses in infant macaques. Aligning our studies with the adjuvanted proteins evaluated in a prime-boost schedule with ALVAC in the ongoing HVTN (HIV Vaccine Trials Network) 702 efficacy trial, we selected the bivalent clade C Env immunogens gp120 C.1086 and gp120 TV1 in combination with the MF59 adjuvant. However, we hypothesized that the adjuvant system AS01, that is included in the pediatric RTS,S malaria vaccine, would promote Env-specific antibody responses superior to those of the oil-in-water MF59 emulsion adjuvant. In a second study arm, we compared two emulsions, glucopyranosyl lipid adjuvant formulated in a stable emulsion (GLA-SE) and 3M-052-SE, containing Toll-like receptor 4 (TLR4) and TLR7/TLR8 (TLR7/8) ligand, respectively. The latter adjuvant had been previously demonstrated to be especially effective in activating neonatal antigen-presenting cells. Our results demonstrate that different adjuvants drive quantitatively or qualitatively distinct responses to the bivalent Env vaccine. AS01 induced higher Env-specific plasma IgG antibody levels than the antigen in MF59 and promoted improved antibody function in infants, and 3M-052-SE outperformed GLA-SE by inducing the highest breadth and functionality of antibody responses. Thus, distinct adjuvants are likely to be required for maximizing vaccine-elicited immune responses in infants, particularly when immunization in infancy aims to elicit both perinatal and lifelong immunity against challenging pathogens such as HIV.

IMPORTANCE Alum remains the adjuvant of choice for pediatric vaccines. Yet the distinct nature of the developing immune system in infants likely requires novel adjuvants targeted specifically at the pediatric population to reach maximal vaccine efficacy with an acceptable safety profile. The current study supports the idea that additional adjuvants for pediatric vaccines should be, and need to be, tested in infants for their potential to enhance immune responses. Using an infant macaque model, our results suggest that both AS01 and 3M-052-SE can significantly improve and

Received 16 June 2018 **Accepted** 30 July 2018

Accepted manuscript posted online 8 August 2018

Citation Phillips B, Van Rompay KKA, Rodriguez-Nieves J, Lorin C, Koutsoukos M, Tomai M, Fox CB, Eudailey J, Dennis M, Alam SM, Hudgens M, Fouda G, Pollara J, Moody A, Shen X, Ferrari G, Permar S, De Paris K. 2018. Adjuvant-dependent enhancement of HIV Env-specific antibody responses in infant rhesus macaques. J Virol 92:e01051-18. https://doi.org/10.1128/JVI.01051-18.

Editor Guido Silvestri, Emory University

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better sustain HIV Env-specific antibody responses than alum. Despite the limited number of animals, the results revealed interesting differences that warrant further testing of promising novel adjuvant candidates in larger preclinical and clinical studies to define the mechanisms leading to adjuvant-improved antibody responses and to identify targets for adjuvant and vaccine optimization.

KEYWORDS HIV, adjuvant, antibody response, pediatric vaccine

The majority of human vaccines mediate protection via the induction of effective antibody responses. Effective antibody responses are dependent on antibody maturation that takes place in germinal centers of secondary lymphoid organs. Follicular T helper (Tfh) cells play a critical role in the activation of germinal center B cells by providing signals that promote somatic hypermutation (1). The activation of Tfh in turn depends on their stimulation by antigen-presenting cells (APCs). In infants, APCs exhibit reduced responses to ligation of pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) (2); frequencies of Tfh are reduced (3, 4), and somatic hypermutation of infant antibodies is limited (5). Thus, the development of vaccines targeted at the pediatric population is faced with several unique challenges. Some of these challenges could be overcome by enhancing vaccine immunogenicity through the use of novel adjuvants. For instance, alum and oil-in-water-based adjuvants, such as squalene-based emulsions, enhance antigen uptake and promote activation and migration of APCs to lymph nodes (LNs) but are not potent inducers of cellular responses against intracellular pathogens, such as human immunodeficiency virus (HIV). In contrast, monophosphoryl lipid A (MPL), a detoxified lipopolysaccharide (LPS) TLR4 agonist, can directly activate the PRR TLR4 on APCs, induce the maturation of APCs, and promote the induction of T helper 1 (Th1) immune responses (6, 7). In infants, however, TLR4 stimulation of APCs, at least *in vitro*, results in poor production of interleukin-12 (IL-12), a cytokine necessary for Th1 responses and NK cell-mediated antiviral immunity (2, 8, 9).

Nonetheless, the results of the RTS,S malaria vaccine trial, that included the adjuvant system AS01, a liposome-based formulation that contains MPL and *Quillaja saponaria* Molina fraction 21 (QS-21), were encouraging. In fact, the WHO and the Malaria Vaccine Implementation Programme will make this RTS,S malaria vaccine available in selected areas of three sub-Saharan African countries for a pilot phase this year through a routine pediatric immunization program in children aged 5 to 17 months (10). Of note, AS01 is included in the licensed herpes zoster vaccine for older adults, a population at the other end of the age spectrum, in which the efficacy of several vaccines is generally also reduced (11, 12).

In vitro studies have repeatedly demonstrated that, among the TLR agonists available for testing, low-molecular-weight imidazoquinolines that activate TLR7/TLR8 (TLR7/8) act as the most potent inducers of IL-12 in infant APCs (9, 13). Imidazoquinolines have not yet been approved as vaccine adjuvants due to concerns about reactogenicity when they are administered systemically. However, chemical modifications of imidazoquinolines and their formulation with alum, squalene, or liposomes (6, 14) resulted in an improved safety profile, at least in animal studies. The potential of TLR7/8 agonists as pediatric vaccine adjuvants was highlighted by a study in infant macaques that compared the immunogenicity of the pneumococcal vaccine (PCV), which is poorly immunogenic in human infants, in the presence and absence of the TLR7/8-based adjuvant 3M-052. The inclusion of 3M-052 in the alum-adjuvanted PCV resulted in a significantly accelerated and enhanced antibody response in infant rhesus macaques (15).

Based on these findings and consistent with our long-term goal of developing an effective HIV vaccine that can be administered along with other routine vaccines in infancy to protect against both postnatal (e.g., through breastfeeding) and lifelong sexual HIV transmission risks, the current pilot study was designed to compare the effects of distinct adjuvants on the induction of HIV Env-specific antibody responses in

TABLE 1 Adjuvant groups

Arm and group[a]	Adjuvant	Adjuvant dose	Injection vol (μl)
1			
A	$Al(OH)_3$	225 μg	250
B	AS01	125 μl[b]	250
C	MF59	125 μl[c]	250
2			
D	$Al(OH)_3$	225 μg	250
E	3M-052-SE	30 μg (with 2% oil)	500
F	GLA-SE	5 μg (with 2% oil)	500

[a]All animals were vaccinated with 15 μg of C.1086 gp120 and 15 μg of TV1 gp120.
[b]A dose of 125 μl of AS01 contains 25 μg of MPL and 25 μg of QS-21 in a liposome-based formulation.
[c]The source material for MF59 was twice concentrated.

infant macaques. Considering the distinct nature of the developing immune system in infants, adjuvants may drive quantitatively or qualitatively distinct responses to a given vaccine in an infant compared to those in adults, suggesting that distinct adjuvants are likely to be required for maximizing vaccine-elicited immune responses in infants. We hypothesized that the adjuvantation of an HIV Env vaccine with AS01 would result in superior antibody responses in infant rhesus macaques compared to responses with alum and also with MF59. MF59 was included because only a few studies have compared AS01 to emulsion adjuvants (16). In parallel, we assessed whether another TLR4-based adjuvant, the synthetic TLR4 ligand glucopyranosyl lipid adjuvant formulated in a stable emulsion (GLA-SE), which has been documented to increase the immunogenicity of influenza vaccine and an experimental Epstein-Barr virus (EBV) vaccine in preclinical studies (17, 18), could also improve upon alum-adjuvanted vaccine responses. In addition, we explored the potential of the TLR7/8-based adjuvant 3M-052 formulated in stable emulsion (3M-052-SE) for enhancing infant Env-specific antibody responses. This pilot study, with the caveat of small group sizes, suggests that adjuvants targeting TLR4 or TLR7/8 can enhance the magnitude and quality of infant Env-specific antibody responses compared to responses with squalene or alum only.

RESULTS

Vaccine regimens and animal groups. We recently demonstrated that HIV Env protein immunizations are effective in inducing robust Env-specific antibody responses in infant macaques (19). In that study, systemic and mucosal immune responses were targeted by administration of Env protein by both the intramuscular (i.m.) and the intranasal (i.n.) routes, with adjuvants of Span85-Tween 80-squalene (STS) adjuvant for i.m. immunizations and with the TLR7/8 agonist R848 for i.n. administration. With the potential of i.n. vaccines to cause adverse side effects in the central nervous system (CNS) being a major safety concern, the current study was designed to evaluate different adjuvants for their potentials to enhance antibody responses to i.m. administered vaccines. The immunogen consisted of two clade C Env proteins, gp120 of C.1086 and gp120 of TV1c8 (here, TV1), because the majority of pediatric HIV infections occur in sub-Saharan Africa where clade C HIV represents the most prevalent clade. The Env from HIV C.1086 was isolated from a transmitted/founder virus, and TV1 Env was derived from an HIV-infected adult in the early chronic-infection stage (20). This bivalent HIV subtype C gp120 protein vaccine has been developed to be similar in immunogenicity and epitope presentation to the clade B and AE Env proteins used in the RV144 HIV vaccine trial that showed moderate efficacy (20–23). In fact, the C.1086 and the TV1 Env immunogens adjuvanted with MF59 in a prime-boost regimen with ALVAC-HIV (vCP2438) are currently being tested in the HVTN (HIV Vaccine Trials Network) 702 HIV vaccine trial in Africa, thus enhancing the translational potential of our study. Consistent with the nature of this study as a pilot study, we did not include additional antigens or vaccine modalities. We had two study arms (Table 1). Arm 1 compared the AS01 ($n = 4$) and MF59 ($n = 4$) adjuvants for their abilities to enhance

Env-specific antibody responses. In arm 2, we tested whether agonists to two distinct TLRs, the intracellular TLR7/8 ($n = 4$) or the surface-expressed TLR4 ($n = 4$), both formulated in a stable emulsion (SE), would differ in their abilities to increase Env-specific antibody responses in infant macaques. As a control, we included two infants in each study arm that received the Env proteins in alum.

Early pediatric HIV vaccine immunogenicity studies in human infants had demonstrated that an accelerated vaccine interval regimen at 0, 2, and 8 weeks of age was able to induce Env-specific antibody responses in neonates (24–27). Our prior studies in the pediatric rhesus macaque model confirmed that accelerated immunization regimens are effective in inducing antibody responses to simian immunodeficiency (SIV) vaccines (28–33). Therefore, we selected a similar vaccine interval in the current study, with immunizations being administered to neonatal/infant macaques at 0, 2, and 6 weeks of age.

Antibody responses in blood were evaluated at week 8, 2 weeks after the last boost when we expected maximum responses, and at week 12 to assess the short-term durability of the responses. A single lymph node biopsy was performed at week 10 to measure gp120-specific B cells. In this pilot study, animals were followed only for 12 weeks, a time deemed sufficient to assess the adjuvant effect on antibody induction. Each group contained four infant macaques, with the exception of the alum vaccine groups that had two animals each. The small group sizes were selected to allow direct, uncorrected comparisons of the antibody responses between novel adjuvant groups that could reach significance in a rank test.

Importantly, none of the adjuvants were associated with unusual inflammation or local skin reaction at the administration site or systemic adverse reactions, such as fever, in any of the animals.

Adjuvant-dependent magnitude of plasma Env-specific IgG antibodies. Independent of the adjuvant, animals in all groups of both study arms developed plasma Env-specific IgG antibody responses to the two Env immunogens (Fig. 1). In arm 1, infant macaques receiving the Env vaccine with AS01 or MF59 had developed higher mean C.1086 gp120-specific plasma IgG antibody levels after the second immunization (week 4) than infants immunized with alum-adjuvanted vaccine, but mean Env-specific plasma IgG antibody concentrations did not differ between the AS01-adjuvanted (mean, 4.2 $\log_{10}$ ng/ml) and MF59-adjuvanted (mean, 3.4 $\log_{10}$ ng/ml) groups. However, at week 8, 2 weeks after the third and final immunization, animals in the AS01 group had 6.5-fold higher Env-specific plasma IgG antibody levels than the animals in the MF59 group ($P = 0.0286$) (Fig. 1). The mean C.1086 gp120-specific plasma IgG antibody concentrations were 35.6- and 5.4-fold higher in AS01-adjuvanted (mean, 5.1 $\log_{10}$ ng/ml) and MF59-adjuvanted (mean, 4.3 $\log_{10}$ ng/ml) animals, respectively, than the level in the alum-adjuvanted animals (mean, 3.5 $\log_{10}$ ng/ml). Despite a slightly sharper decline in Env-specific plasma IgG antibodies from week 8 to 12 in the AS01 group (mean concentration at week 12, 4.4 $\log_{10}$ ng/ml) than in the MF59 and alum groups (mean week 12 concentrations of 3.9 $\log_{10}$ ng/ml and 3.0 $\log_{10}$ ng/ml, respectively), these infants still had the highest Env-specific plasma IgG antibody levels in arm 1 (Fig. 1). Responses to TV1 gp120 immunogen were almost 1 log lower but followed a similar trend (Fig. 1B and C). TV1 gp120-specific plasma IgG antibody levels were highest in animals receiving the AS01-adjuvanted vaccine. The magnitude of TV1-specific plasma IgG in the AS01 group exceeded the responses induced by MF59-adjuvanted vaccine at 2 and 4 weeks after the third immunization ($P = 0.0286$), but by week 12, 6 weeks after the last immunization, TV1-specific IgG levels were comparable between the AS01 and MF59 groups (Fig. 1B).

In arm 2, differences in Env-specific plasma IgG antibody concentrations depending on the vaccine adjuvant GLA-SE or 3M-052-SE used became apparent only after the third immunization. Infants vaccinated with 3M-052-SE-adjuvanted vaccine had developed the highest C.1086 gp120-specific plasma IgG antibody concentrations of all groups (Fig. 1A), with mean Env-specific plasma IgG antibody concentrations being about 1 log higher ($P = 0.0286$) than responses induced by GLA-SE-adjuvanted vaccine

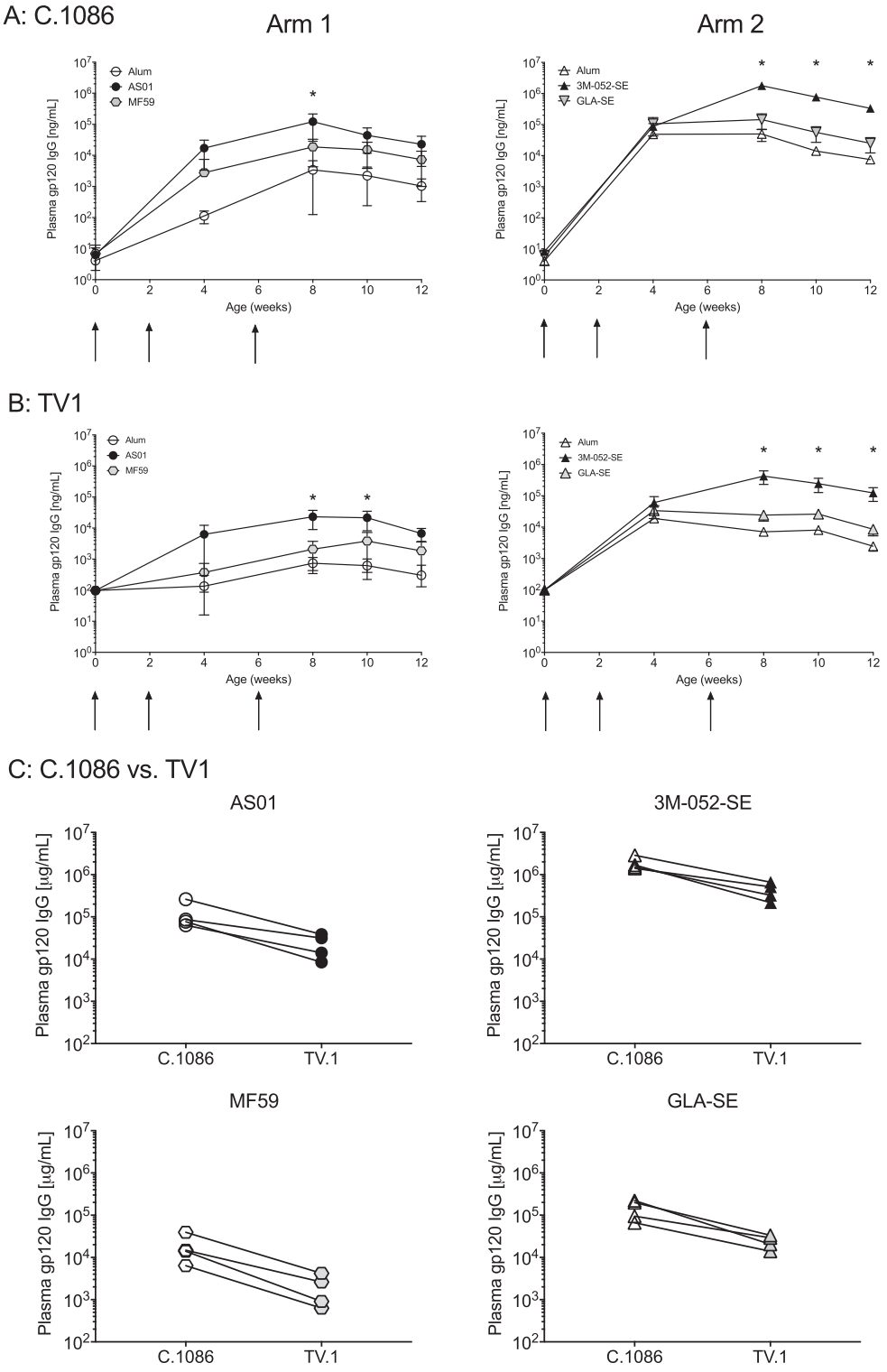

FIG 1 Env-specific plasma IgG antibodies. (A and B) The kinetics of C.1086- and TV1-specific plasma IgG antibodies induced by vaccination with Env protein given in the presence of different adjuvants. Animals in arm 1 received the vaccine with alum, AS01, or MF59, as indicated. In arm 2, the vaccine was adjuvanted with alum, 3M-052-SE, or GLA-SE, as indicated. Mean values ± SD are graphed. Black arrows underneath the *x* axes indicate the immunization time points at weeks 0, 2, and 6. The asterisk (*) indicates statistically significant differences between the gp120-specific antibody concentrations induced by AS01 versus those induced by MF59-adjuvanted vaccines or by 3M-052-SE versus GLA-SE-adjuvanted vaccines (Mann-Whitney test; $P = 0.0286$). (C) The data illustrate the reduced magnitude of plasma IgG antibodies to gp120 of TV1 compared to the level of the gp120 protein of C.1086 at week 8, 2 weeks after the last immunization. Responses by the same animal in each of the adjuvant groups are represented by a connecting line.

and 2 logs higher than responses elicited by alum-adjuvanted vaccine (Fig. 1A). Similar to our findings in arm 1, responses to the TV1 Env immunogen were of lower magnitude than the response to the C.1086 Env (about 0.5 log) (Fig. 1C), but the same hierarchy was observed, with plasma IgG concentrations being consistently higher after the third and last immunization in animals that received the vaccine in the presence of the 3M-052-SE adjuvant ($P = 0.0286$) (Fig. 1B).

Breadth and epitope specificity of antibody responses. Although the majority of pediatric HIV infections occur in sub-Saharan Africa where clade C is most prevalent, a vaccine to prevent breast milk transmission of HIV should ideally provide protection globally against multiple, different HIV strains. Applying the binding antibody multiplex assay (BAMA), we tested the specificity of plasma IgG antibodies not only to the vaccine immunogens C.1086 and TV1 but also to the gp120 proteins of the clade B strain MN and the clade AE strain A244, the Env proteins used in the RV144 human HIV vaccine trial. While several animals in various vaccine groups had detectable cross-clade-specific antibodies, only the infant macaques vaccinated with 3M-052-SE-adjuvanted HIV Env had developed cross-reactive antibodies to clade AE Env and clade B Env at week 8 that were comparable in magnitude to those induced to clade C Envs (Fig. 2A). Consistent with a decline in the overall antibody response from week 8 to week 12, cross-clade specific antibody levels also declined. The persistence of clade B and clade AE cross-reactive antibodies was highest in the 3M-052-SE group (Fig. 2B). These data suggested that the breadth of the Env-specific IgG response was most effectively enhanced by the 3M-052-SE adjuvant. In both study arms, infants receiving alum-adjuvanted vaccine exhibited the lowest breadth in their Env responses.

We observed the same hierarchy of responses when we assessed epitope specificity across clades at week 8 using BAMA. In each study arm, alum-adjuvanted vaccine induced the lowest responses, and responses were highest in the AS01 group of arm 1 and in the 3M-052-SE group in study arm 2. Independent of the vaccine adjuvant, plasma IgG responses were strongest to the consensus clade C V3 region, followed by responses to the V1V2 epitope of C.1086 (Fig. 3). The same epitopes were targeted when clade B antigens were tested (Fig. 3). At week 12, 6 weeks after the last immunization, we performed peptide array mapping to determine epitope specificity. The data confirmed that V3-specific antibodies dominated the antibody response (Fig. 4). Plasma IgG antibodies also recognized epitopes in the C5 and C1 regions. It should be noted that responses to the V2 region were detectable only against V2 of C.1086 and not against V2 of the TV1 immunogen, whereas responses against the C2.4 p88-p89 epitope were more pronounced for the TV1 immunogen (Fig. 4).

Avidity of plasma Env-specific antibodies is influenced by adjuvant selection. Antibody avidity is an important measurement of the strength with which an antibody can interact with its relevant antigen. Thus, we wanted to test whether the observed differences in the magnitudes of the plasma IgG responses that were dependent on the vaccine adjuvant would be reflected in antibody avidity, too. As expected, among the adjuvant groups tested in each study arm, antibody avidity was lowest in the alum group (Fig. 5). In arm 1, plasma IgG antibodies specific for the C.1086 immunogen exhibited avidity similar to that in animals of the AS01- and MF59-adjuvanted vaccine groups (Fig. 5). Comparable antibody avidity to C.1086 gp120 was also observed when we compared the avidity scores in animals vaccinated in the presence of 3M-052-SE or GLA-SE in arm 2 (Fig. 5).

The antibody avidity to the TV1 gp120 immunogen appeared to be slightly lower than the avidity of plasma IgG antibodies to C.1086, and although not statistically significant, this trend was observed in both study arms in each of the vaccine groups (Fig. 6). The reduced antibody avidity to the TV1 gp120 revealed potential differences in adjuvant activity. Thus, there was a trend toward lower antibody avidity in infants of the MF59 group than in those in the AS01 group in arm 1. In arm 2, infants that received 3M-052-SE-adjuvanted vaccine had plasma antibodies with significantly higher avidity ($P = 0.0286$) to TV1 gp120 than infants in the GLA-SE group (Fig. 5 and 6).

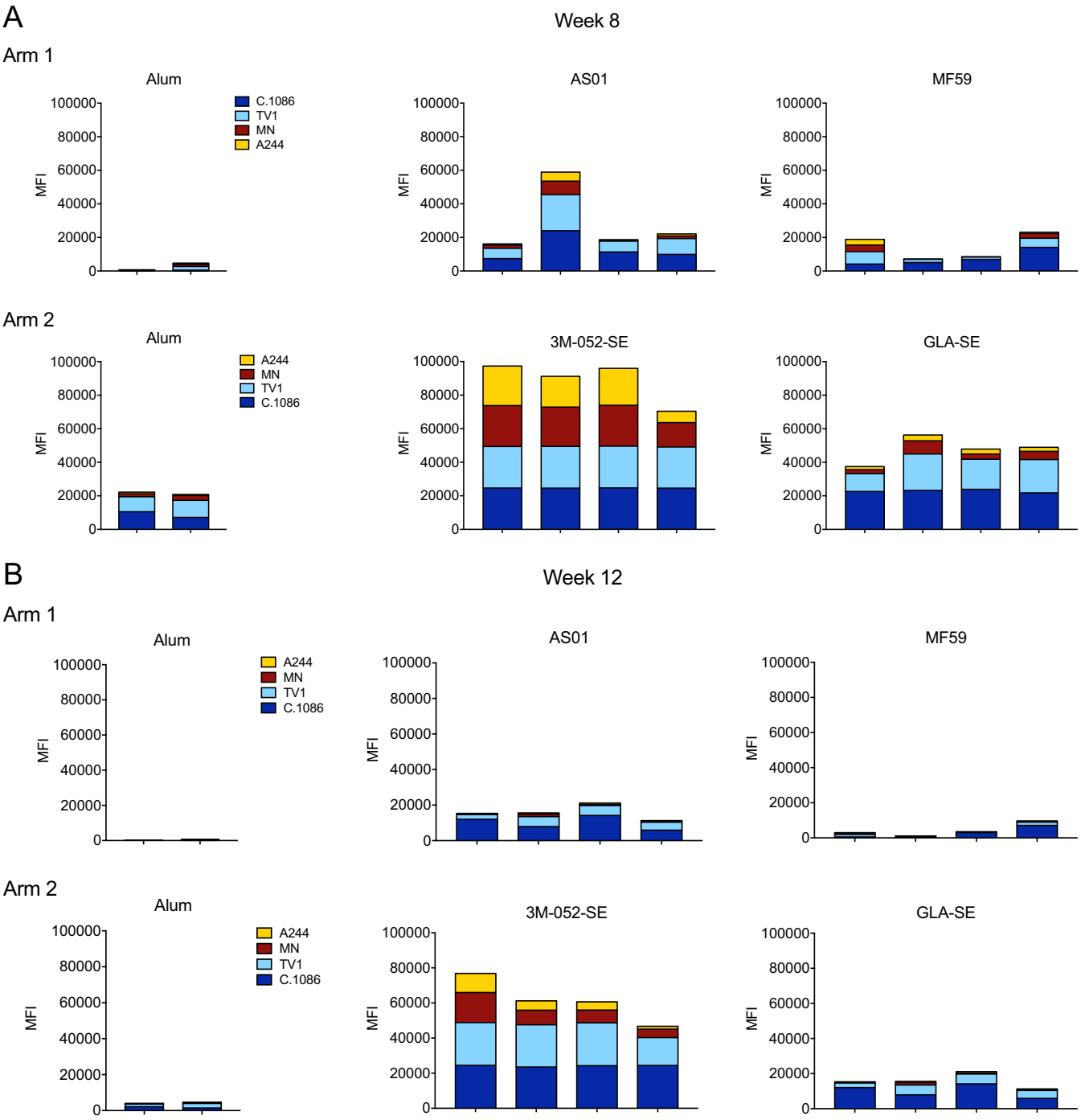

FIG 2 Cross-clade Env-specific plasma IgG antibodies. Plasma IgG antibodies were tested by BAMA at week 8 (A) and week 12 (B) for specificity to gp120 of the two vaccine immunogens C.1086 and TV1 and for cross-clade specificity to gp120 of the clade B strain MN and the clade AE strain A244. Shown are MFI values, with each bar representing a single animal in each of the vaccine groups. Each bar represents the sum of individual responses measured to a specific gp120 protein, with each Env depicted by a different color, as indicated.

Impact of adjuvants on ADCC function. In the human RV144 HIV vaccine trial, antibody-mediated cytotoxicity (ADCC) was identified as one of the immune correlates associated with reduced HIV infection risk (22, 34, 35). We therefore evaluated ADCC function at week 8, when Env-specific plasma IgG antibodies were at their peak levels, and after the initial decline at week 12 (Fig. 1). ADCC activity was measured using target cells coated with either gp120 C.1086 or with gp120 TV1. In arm 1, the infant macaques

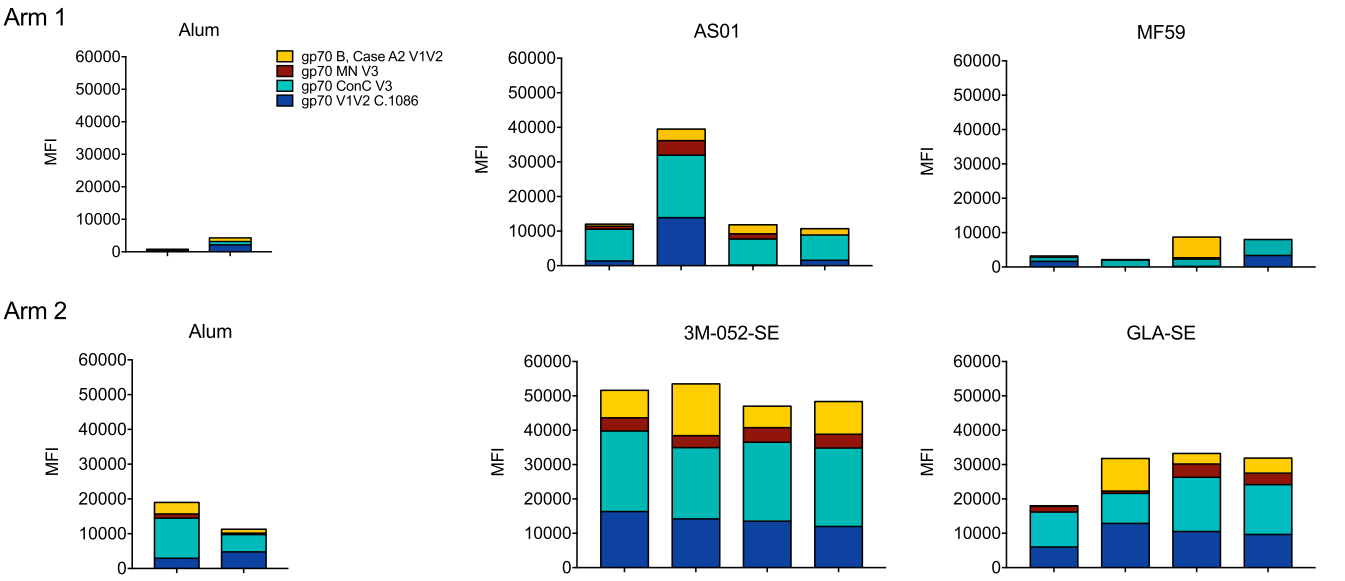

FIG 3 Epitope specificity. Plasma IgG antibodies were tested by BAMA at week 8 for specificity to gp70 V1V2 C.1086, gp70 ConC3, gp70 MN V3, and gp70 B.Case A2 V1V2. Shown are MFI values, with each bar representing a single animal in each of the vaccine groups. Each bar represents the sum of individual responses measured to a specific protein epitope, with each epitope depicted by a different color, as indicated.

of the AS01 and the MF59 groups exhibited comparable ADCC activities, as measured by endpoint titer and maximum granzyme B activity, to the gp120 of C.1086. However, ADCC activity against TV1 gp120-coated target cells trended lower in the MF59 than in the AS01 group (Fig. 7A). By week 12, none of the animals in the MF59 group exerted ADCC activity to TV1 gp120-coated target cells, whereas ADCC activity was still detectable in two of four infant macaques of the AS01 group (Fig. 7B). Alum-adjuvanted control animals in arm 1 had only very low or no detectable ADCC activity (Fig. 7). In contrast, ADCC activity was detectable in the alum controls of the second study arm (Fig. 8). It is possible that the commercially obtained alum used in arm 2 contained some contaminants that may have enhanced immune responses in an unspecific way, in contrast to the alum utilized in arm 1 that was provided by GlaxoSmithKline (GSK). However, these findings should not be overinterpreted as we had only two alum controls in each study arm. The main comparisons were between AS01 and MF59 in arm 1 and between 3M-052-SE and GLA-SE in arm 2.

The highest ADCC activity of plasma IgG antibodies in vaccinated infants of arm 2 was observed when the vaccine was combined with the 3M-052-SE adjuvant (Fig. 8A and B). Even at week 12, the highest dilution of plasma at which ADCC activity was still detectable was higher than that in any other vaccine group. Based on endpoint titer dilution, the ADCC activity in infants of the GLA-SE group was about 0.5 to 1 $\log_{10}$ lower ($P = 0.0286$) (Fig. 8). Maximum granzyme B activity, however, varied widely among the arm 2 animals, irrespective of the adjuvant, and therefore median granzyme B activities did not differ between these two adjuvant groups (Fig. 8). Again, vaccine-induced ADCC activity was higher against the gp120 of C.1086 than against target cells coated with the TV1 gp120 protein (Fig. 8C).

Vaccine adjuvants alter tier 1 neutralizing antibody responses. Although vaccination with Env gp120 monomers is not expected to induce strong neutralizing antibody responses, tier 1 neutralizing antibodies can be induced (19). Therefore, we tested whether the neutralizing antibody responses differed depending on the vaccine adjuvant. In arm 1, clade C tier 1 virus MW965 neutralizing antibodies were poorly induced (median 50% inhibitory concentration [IC_{50}] below 100) when the vaccine was adjuvanted with MF59 or alum (Fig. 9). In contrast, inclusion of AS01 as adjuvant resulted in the induction of tier 1 virus neutralizing antibodies although median titers at peak plasma IgG responses (week 8) did not exceed a titer of 500. Both 3M-053-SE

Linear Epitope Mapping

Study Arm	Adjuvant	Animal No.	1086.C Epitopes												
			C1.1 p32-37	C1.2 p39-41	V2 p53-55	C2.1 p65-67	C2.2 p70-73	C2.3 p83-86	C2.4 p88-89	V3 p98-103	V4.1 p123-124	V4.2 p132-132	V5 p146-149	C5.1 p152-153	C5.2 p156-162
1	Al(OH)3	45934	3.02	2.78	0.53	-0.90	0.90	0.69	0.62	0.93	0.02	0.55	0.44	2.84	2.90
		45935	2.13	1.27	1.30	0.39	3.09	-0.35	0.33	4.74	0.70	0.19	1.70	0.62	-0.24
	AS01B	45937	0.96	0.21	2.62	1.60	4.91	-1.62	0.54	3.40	1.39	0.51	1.10	0.41	0.53
		45939	2.03	1.43	6.44	0.28	1.93	-0.65	0.13	8.21	1.89	-0.90	0.79	0.19	4.59
		45941	0.39	0.69	2.01	0.17	1.17	-0.84	-0.59	6.51	1.00	0.55	0.01	0.45	1.18
		45943	3.92	-0.05	0.90	0.00	1.68	2.49	0.50	5.19	1.35	-0.76	-0.54	-1.27	2.27
	MF59	45906	3.88	2.03	3.76	0.67	1.24	0.22	-1.01	6.16	0.52	-0.66	1.09	2.53	0.87
		45909	1.99	1.97	3.44	1.50	5.00	-1.53	0.60	2.26	2.31	0.10	1.29	2.40	1.48
		45910	0.84	0.51	0.37	0.93	0.39	2.81	0.52	3.99	0.10	0.31	-0.99	-0.09	2.41
		45914	1.03	0.70	2.05	1.25	4.21	-1.03	0.50	3.21	1.66	0.18	1.05	1.58	0.82
2	Alum	45717	6.17	0.40	-0.98	3.66	-0.36	3.86	1.59	7.77	-0.63	-0.09	4.53	1.28	2.44
		45718	3.33	0.88	3.18	2.80	1.36	3.25	0.72	6.79	0.16	0.40	3.39	4.21	4.02
	3M-052	45838	9.18	2.56	2.95	1.10	1.41	0.24	0.43	9.97	1.19	-0.09	6.68	6.27	10.65
		45840	8.73	1.47	5.23	5.98	0.93	0.57	-0.14	8.83	1.02	-0.61	5.36	2.73	6.29
		45847	9.28	1.81	3.60	9.40	-0.22	3.02	0.89	9.82	2.35	-0.31	5.88	7.03	7.62
		45851	3.04	1.35	2.73	1.45	2.96	-0.52	0.64	7.11	0.93	-0.21	3.04	5.09	4.41
	GLA-SE	45687	5.21	1.63	3.99	0.20	0.43	4.98	0.23	8.85	0.29	-0.06	3.61	6.79	10.45
		45688	0.53	-0.03	2.01	0.02	3.88	-2.05	0.72	4.18	1.38	1.15	1.13	0.89	1.86
		45723	5.76	1.03	2.59	-1.03	1.04	1.32	0.27	8.00	0.27	-0.05	1.66	1.75	4.59
		45724	1.75	2.03	3.01	1.99	3.41	3.48	1.29	5.68	2.38	0.46	0.93	2.07	3.79

Study Arm	Adjuvant	Animal No.	TV1 Epitopes												
			C1.1 p32-37	C1.2 p39-41	V2 p53-55	C2.1 p65-67	C2.2 p70-73	C2.3 p83-86	C2.4 p88-89	V3 p98-103	V4.1 p123-124	V4.2 p132-132	V5 p146-149	C5.1 p152-153	C5.2 p156-162
1	Al(OH)3	45934	3.73	2.51	1.22	-0.61	-0.06	0.68	2.22	2.00	-0.47	2.76	1.72	2.84	2.95
		45935	2.53	1.27	1.95	0.08	1.69	-0.79	1.08	4.51	1.19	1.22	2.87	0.62	1.01
	AS01B	45937	0.88	0.60	2.14	1.34	3.77	-1.75	-0.09	3.20	1.75	1.58	0.44	0.41	1.40
		45939	5.24	1.72	1.86	0.04	1.39	-0.80	4.02	6.13	0.94	-0.45	-0.63	0.19	5.57
		45941	0.76	1.65	1.15	0.02	0.78	-0.33	2.93	5.44	0.29	0.36	0.10	0.45	3.13
		45943	3.41	1.01	1.05	0.18	-0.03	1.32	3.55	8.63	-0.41	0.10	1.63	-1.27	7.48
	MF59	45906	7.60	1.61	0.15	0.03	0.97	0.00	2.28	4.89	-0.31	-0.17	0.64	2.53	0.62
		45909	1.91	1.68	2.28	1.43	4.02	-1.81	-0.37	2.84	1.76	1.62	0.78	2.40	2.13
		45910	1.61	0.77	-0.70	1.10	-0.12	2.12	4.55	6.93	-0.28	1.27	-0.42	-0.09	5.03
		45914	1.11	0.63	1.51	0.87	2.57	-1.81	1.24	3.46	1.81	2.38	0.13	1.58	1.40
2	Alum	45717	5.80	1.05	1.70	3.08	-1.28	2.47	5.58	5.97	-0.15	0.57	6.13	1.28	5.45
		45718	2.57	0.73	1.48	2.63	0.19	0.73	3.39	6.10	0.71	0.52	5.32	4.21	7.22
	3M-052	45838	9.55	2.76	1.56	1.38	0.49	0.02	3.71	10.08	0.87	0.42	11.04	6.27	10.09
		45840	6.64	0.51	1.98	5.84	0.48	0.35	6.10	8.44	0.89	0.65	6.03	2.73	8.58
		45847	9.84	1.65	1.22	8.78	0.00	-0.76	4.74	9.27	1.56	0.43	7.26	7.03	10.19
		45851	2.79	0.11	1.84	1.50	2.15	-0.17	1.46	6.92	1.12	1.46	3.79	5.09	6.39
	GLA-SE	45687	3.31	1.12	0.64	0.51	-0.35	4.04	5.58	8.10	-0.96	-2.13	4.52	6.79	7.23
		45688	0.62	0.08	1.73	0.78	3.12	-1.83	1.98	3.45	2.09	0.66	0.16	0.89	1.12
		45723	4.75	0.77	0.99	0.46	0.44	0.63	3.93	5.48	0.18	0.16	1.91	1.75	8.95
		45724	1.88	2.35	3.08	1.83	2.69	2.55	2.99	4.08	2.07	2.45	0.64	2.07	7.04

Legend	Log2 MFI
	<0 to 2
	>2 to 4
	>4 to 6
	>6 to 8
	>8 to 10
	>10

FIG 4 Plasma IgG linear epitope mapping by peptide microarray. The heat map shows binding values to specific epitopes of the C.1086 Env (top) or the TV1 Env (bottom) for each animal in each of the vaccine groups in the two different study arms. The legend illustrates that the binding magnitude increases from less than $\log_2$ MFI (white) to >10 $\log_2$ MFI (red).

and GLA-SE were able to increase tier 1 neutralizing antibody titers compared to the titers with alum in arm 2. Infant macaques vaccinated with Env protein adjuvanted with 3M-052-SE had the highest neutralizing antibody titers at week 8 (median value, 3,083), and despite a decline by week 12, neutralizing antibodies persisted and remained higher than those induced by GLA-SE- or alum-adjuvanted vaccine (Fig. 9). Detectable tier 2 autologous vaccine virus C.1086 neutralizing antibodies were not induced by any of the vaccine groups (data not shown).

Env-specific B cells in lymph nodes. Antibody responses are primed and undergo maturation in lymph nodes when antigen-specific B cells receive the necessary signals from follicular T helper cells. gp120-specific B cell frequencies were measured in axillary lymph nodes collected 4 weeks after the last immunization. Consistent with the overall higher C.1086 gp120-specific plasma IgG antibody concentrations, the 3M-052-SE group was the only vaccine group in which all animals had detectable gp120-specific B cells in peripheral lymph nodes (Fig. 10).

DISCUSSION

An effective HIV vaccine to prevent breast milk transmission of HIV needs to be administered with an accelerated schedule and to induce protective immune responses when the infant's immune system is still maturing. Ideally, this pediatric vaccine response could be boosted later in life to provide durable and mature immunity prior to sexual debut. Both innate and adaptive immune functions are distinct in infants from

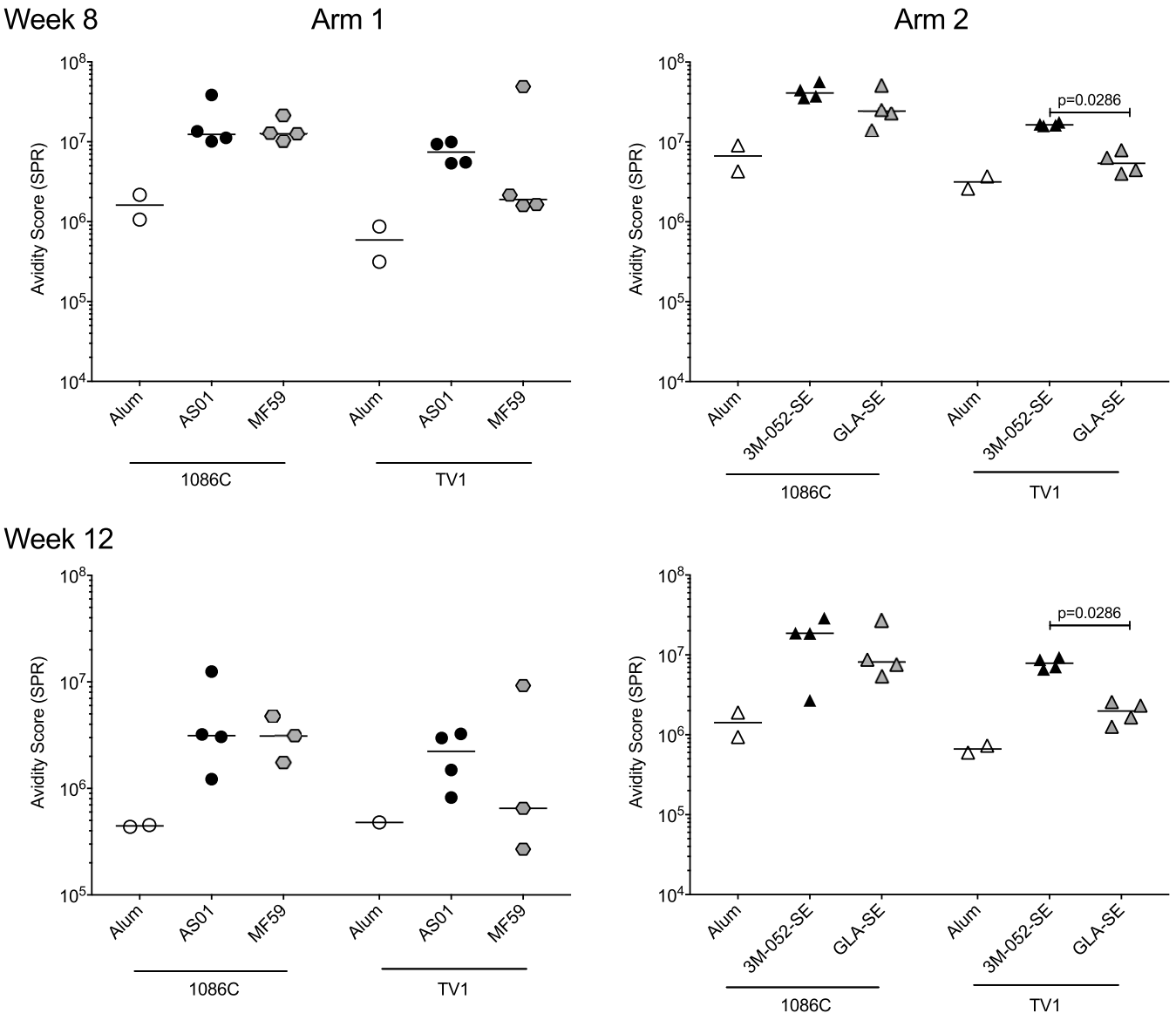

FIG 5 Antibody avidity. The avidity of plasma IgG antibodies specific for C.1086 or TV1 was measured by SPR at week 8 and at week 12. The avidity score for each animal is shown, with median values indicated by a horizontal bar for each group. Statistically significant differences in avidity scores were determined by Mann-Whitney test.

those in adults, due to limited antigen exposure and the need to simultaneously enable the establishment of normal beneficial microbial flora and educate the immune system to recognize pathogenic microbes. To overcome these challenges and optimize infant vaccine-induced immune responses, adjuvants are likely to play a critical role.

In the current study, we evaluated adjuvants that were selected based on their translational potential (AS01 and MF59) and on their capacity to specifically enhance pediatric vaccine responses (3M-052-SE). Toward the goal of developing a pediatric HIV vaccine, these adjuvants were tested for their abilities to induce Env-specific antibodies with Fc effector function in response to a bivalent HIV Env vaccine consisting of C.1086 gp120 and TV1 gp120 proteins. We acknowledge that prior studies in adults have demonstrated that an HIV vaccine consisting of only Env protein will not be sufficient to prevent HIV infection (36, 37). The focus of our study instead was to compare different adjuvants for their abilities to improve Env-specific antibody responses in infants. However, in view of ongoing clinical trials, the bivalent Env vaccine in the current study contains the same Env proteins currently being tested in the HVTN 702

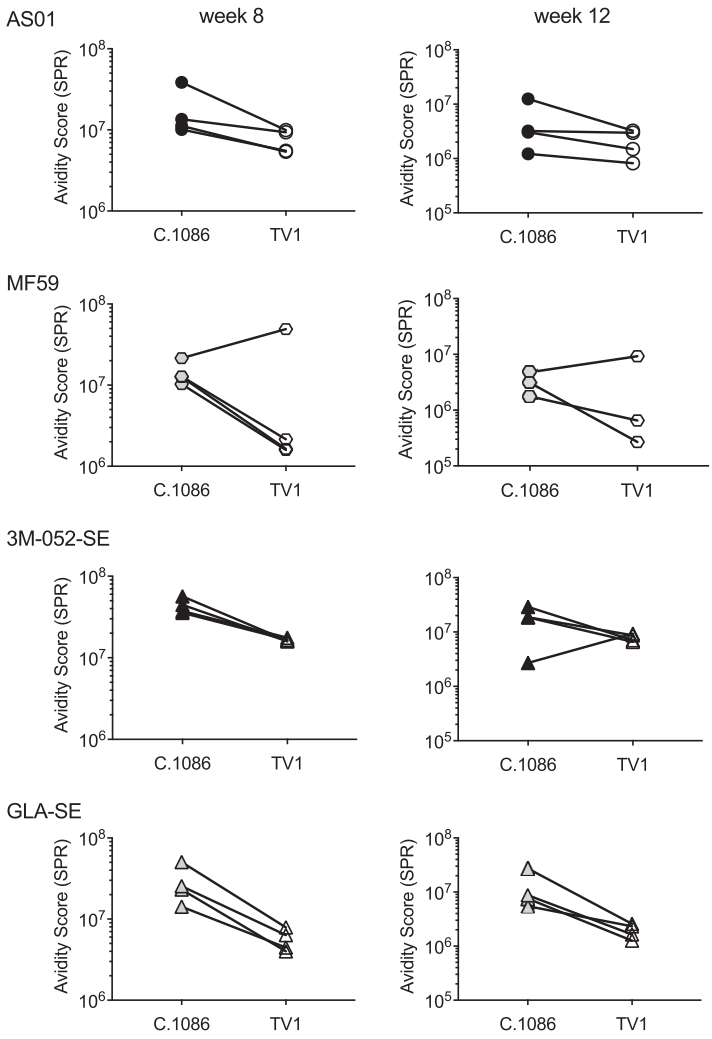

FIG 6 Avidity differences of plasma IgG antibodies to the different Env immunogens. The graphs show the avidity scores of plasma IgG antibodies for each animal in each of the vaccine groups measured against C.1086 gp120 (filled symbols) or TV1 gp120 (open symbols). The avidity scores for the distinct immunogens are connected by a line for individual animals to illustrate the reduced avidity to TV1 compared to that to C.1086 gp120.

HIV vaccine trial in adults. The adjuvant for the bivalent Env in the HVTN 702 trial is MF59. It should be noted that MF59 was demonstrated to be superior to alum in enhancing gp120 protein vaccine-induced antibody responses in human neonates almost 20 years ago in the first pediatric HIV vaccine trial (Pediatric AIDS Clinical Trials Group 230) (27). Yet alum has remained the adjuvant of choice for pediatric vaccines due to the excellent safety record of licensed vaccines. The results of the current study in infant macaques affirmed that MF59 enhances the magnitude of Env-specific plasma IgG responses compared to those with alum. Infant macaques vaccinated with bivalent Env and AS01 had even higher Env-specific plasma IgG antibody levels than infants that received the vaccine with MF59 and exhibited improved antibody function. Thus, in arm 1, avidity and ADCC activity, antibody features associated with protective efficacy in the human RV144 HIV vaccine trial (22, 34, 35), were highest when AS01 was included in the Env vaccine. The fact that AS01 is already included in a pediatric vaccine, the RTS,S malaria vaccine used in the same geographic region where HIV is highly prevalent, underscores its translational potential for pediatric HIV vaccines.

In the second arm of the current study, we tested GLA, an adjuvant that is chemically synthesized and targets TLR4 and is in phase 1/2 clinical testing. GLA was formulated

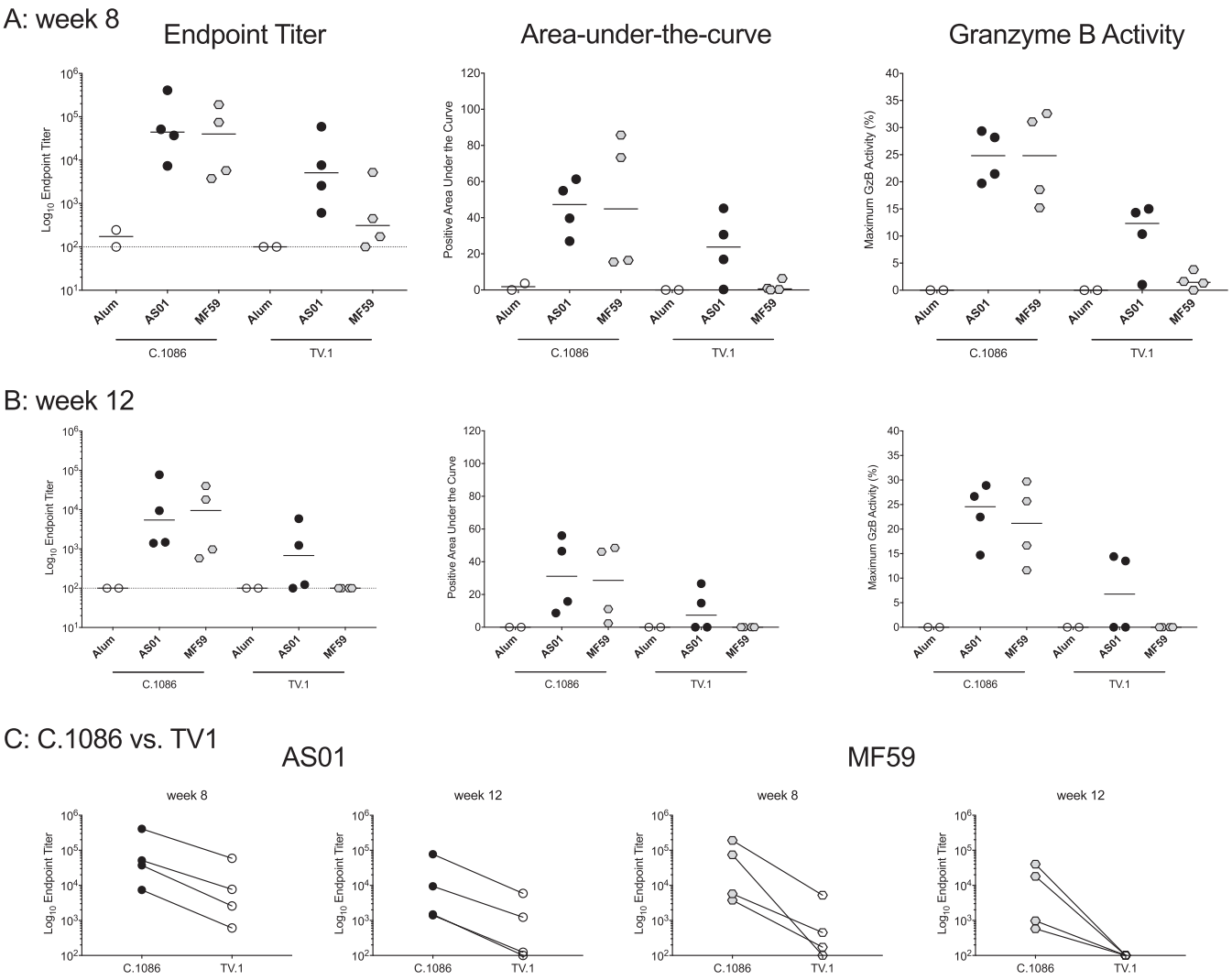

FIG 7 ADCC activity of plasma antibodies in arm 1. ADCC activity was measured at week 8 (A) and week 12 (B). The left graphs show the $\log_{10}$ endpoint dilution titer at which ADCC activity was detectable. The area under the concentration-time curve of serial plasma dilutions used to determine the endpoint titer and maximum granzyme B activity are also shown. Each symbol represents an individual animal; median values for each group are indicated by horizontal bars. Comparisons of statistical differences in endpoint titers between two different adjuvant groups were determined by a Mann-Whitney test. (C) Endpoint titers for individual animals to C.1086 gp120-coated target cells (filled symbols) or TV1 gp120-coated target cells (empty symbols) at weeks 8 and 12 for AS01 and MF59.

in stable emulsion (GLA-SE), while AS01 is formulated in liposomes and contains MPL and QS-21. Therefore, to evaluate the performance of different TLR ligands in the same formulation, we compared GLA-SE to 3M-052-SE. We chose the TLR7/8 agonist 3M-052 because TLR7/8 agonists are uniquely able to activate neonatal and infant APCs (2, 9). Indeed, our results demonstrated that the highest breadth and functionality were observed when infant macaques were vaccinated with the 3M-052-SE-adjuvanted Env protein (38, 39). Our finding that 3M-052-SE outperformed GLA-SE is consistent with previous studies documenting the potency of TLR7/8-based adjuvants for pediatric vaccines. This includes the aforementioned vaccination of neonatal macaques with PCV in the absence and presence of 3M-052 (15) and a study in which infant macaques vaccinated with an inactivated influenza vaccine conjugated to the TLR7/8 agonist R848 developed enhanced and sustained antibody responses compared to responses of infants receiving unadjuvanted vaccine (38, 39). In contrast to alum or oil-in-water emulsions like MF59, adjuvants based on or incorporating TLR ligands can directly activate innate responses and thereby improve priming and the induction of adaptive immune responses by vaccination. The differences observed between GLA-SE and

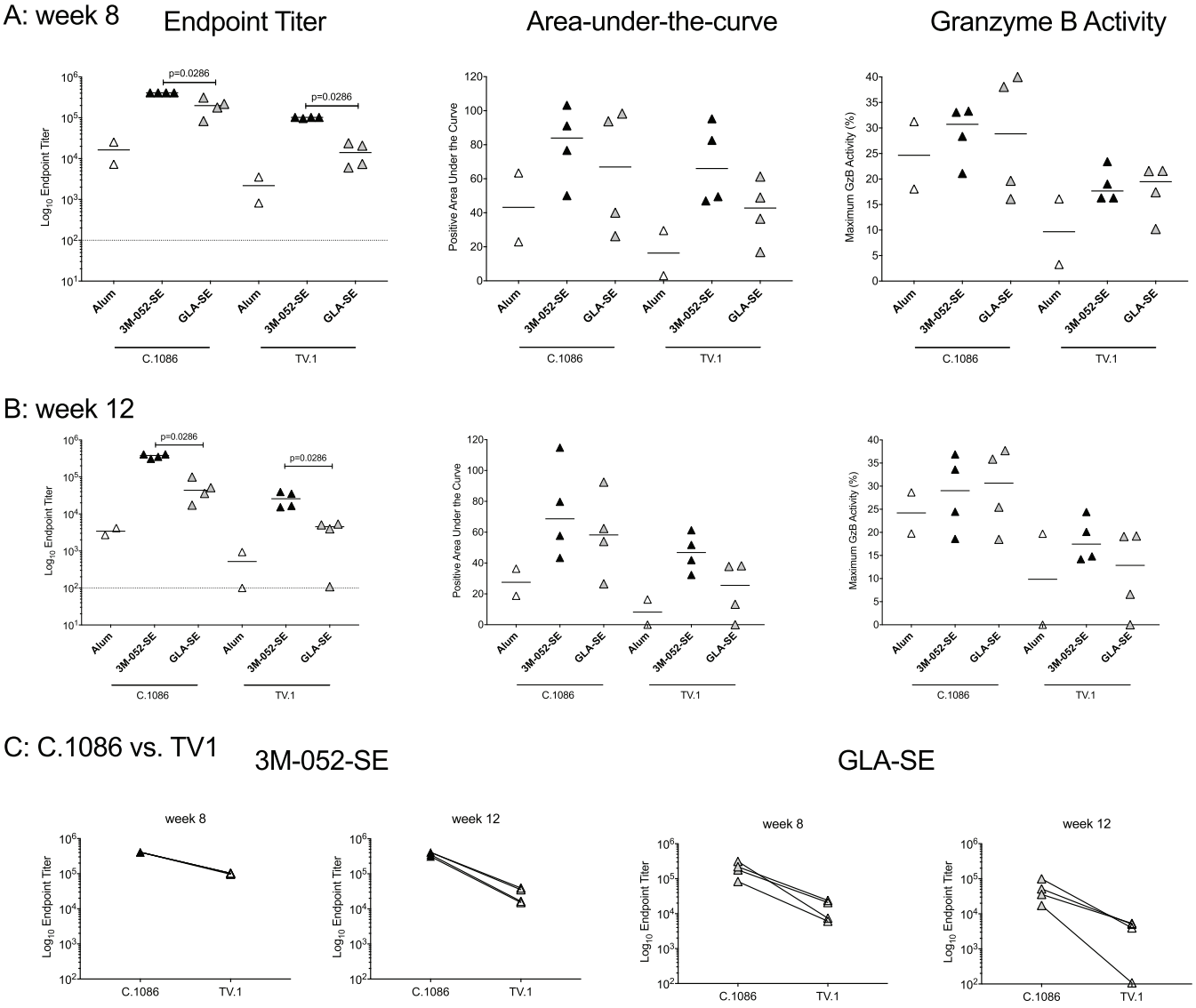

FIG 8 ADCC activity of plasma antibodies in arm 2. ADCC activity was measured at week 8 (A) and week 12 (B). The left graphs show the $\log_{10}$ endpoint dilution titer at which ADCC activity was detectable. The area under the concentration-time curve of serial plasma dilutions used to determine the endpoint titer and maximum granzyme B activity are also shown. Each symbol represents an individual animal; median values for each group are indicated by horizontal bars. Comparisons of statistical differences in endpoint titers between two different adjuvant groups were determined by a Mann-Whitney test. (C) Endpoint titers for individual animals to C.1086 gp120-coated target cells (filled symbols) or TV1 gp120-coated target cells (empty symbols) at weeks 8 and 12 for 3M-052-SE and GLA-SE.

3M-052-SE might be related to the distinct expression patterns of TLR4 and TLR7/8 in immune cells. Both TLR4 and TLR7/8 are expressed by monocyte/macrophages and conventional dendritic cells, but TLR7/8 is also expressed by plasmacytoid dendritic cells (40). Furthermore, depending on the cell type, the specific TLR ligand, and its concentration and formulation, different intracellular signaling pathways will be activated that will result in specific gene and protein expression patterns (41–43).

The superior adjuvant activities of AS01 in arm 1 and of 3M-052-SE in arm 2 were underscored by the analysis of Env-specific antibody responses to the TV1 immunogen that appeared to be less immunogenic than gp120 of C.1086. These differences in immunogenicity between the two distinct clade C Envs was unexpected because in both the initial rabbit studies and a subsequent adult rhesus macaque study, purified gp120 of TV1 and C.1086 induced plasma IgG antibodies of similar magnitudes and with comparable avidities (20, 21). However, it has been reported previously that

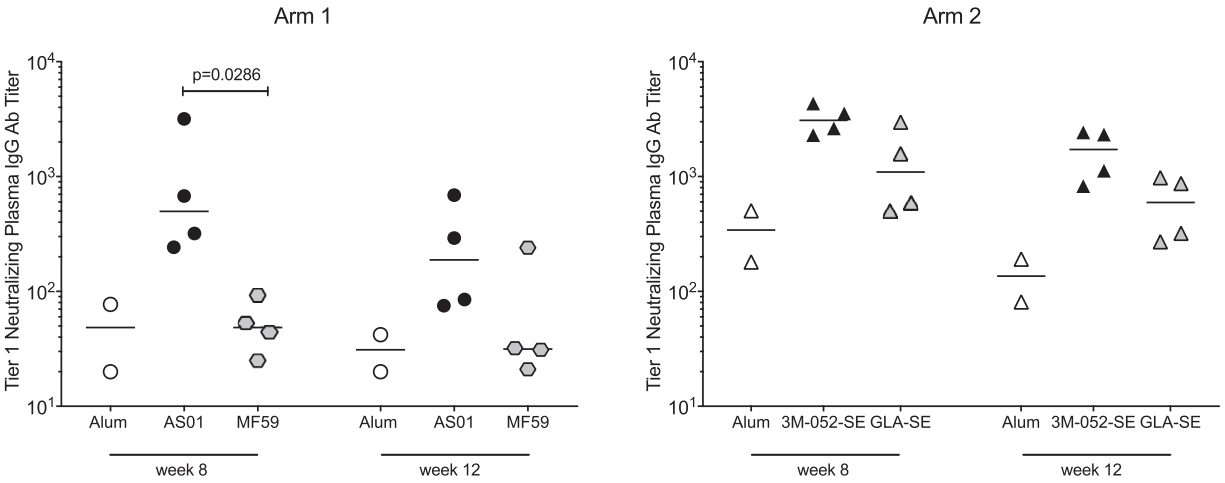

FIG 9 Tier 1 neutralizing plasma antibodies. Neutralization of tier 1 clade C viruses by plasma was measured in TZM.bl cells using MW965.LucR.T2A.ecto/293T at weeks 8 and 12. The reduction of luciferase reporter gene expression after a single round of infection was determined, and the plasma dilution or antibody concentration that caused a 50% reduction in relative light units (RLUs) compared to the RLU values for the virus control wells after subtraction of control RLU values are reported. The preimmunization time point (week 0) was used to measure background levels of neutralization. Each symbol represents an individual animal; median values for each group are indicated by horizontal bars. Comparisons of statistical differences in endpoint titers between two different adjuvant groups were determined by Mann-Whitney test.

antibodies elicited to C.1086 Env exhibit better binding to V1V2 epitopes than anti-TV1 Env antibodies and that the V1V2 recognition by C.1086 Env-specific antibodies is highly similar to that observed in response to the AE.A244 Env immunogen that was included in the RV144 trial (21, 22). Consistent with this finding, linear epitope mapping revealed reduced binding of plasma IgG antibodies to the V2 p53-p55 epitope of TV1 Env compared to that of the C.1086 Env.

Overall, the data presented support the idea that adjuvants for pediatric vaccines should be, and need to be, tested in infants for their potential to enhance infant immune responses. Of interest, our data suggest that both AS01 and 3M-052-SE can significantly improve HIV Env-specific antibody responses in infant macaques and sustain these responses better than alum.

The results of this pilot preclinical study revealed interesting differences between adjuvants that should lead to further exploration of the mechanisms leading to adjuvant-improved antibody responses and to identification of targets for further adjuvant and vaccine optimization.

MATERIALS AND METHODS

Animals. SIV- and type D retrovirus-negative newborn Indian-origin rhesus macaques (*Macaca mulatta*) were hand-reared in the nursery of the California National Primate Research Center ([CNPRC]

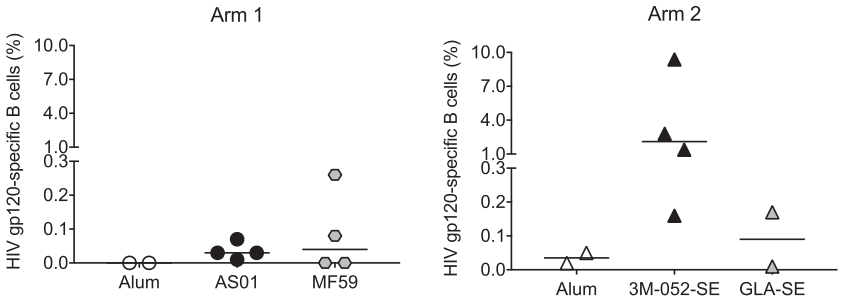

FIG 10 Antigen-specific B cells in lymph nodes. Frequencies of gp120-specific B cells were measured by flow cytometric analysis in lymph node cell suspensions at week 10, 4 weeks after the last immunization. Each symbol represents an individual animal, and horizontal bars represent median group values. Note that due to limited cell numbers, gp120-specific B cells could be quantified in only 2 of 4 infants of the GLA-SE group.

Davis, CA) in accordance with the American Association for Accreditation of Laboratory Animal Care standards. The *Guide for the Care and Use of Laboratory Animals* by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council (44), and the *International Guiding Principles for Biomedical Research Involving Animals* (45) were strictly adhered to. All protocols were reviewed and approved by the University of California at Davis Institutional Animal Care and Use Committee prior to the initiation of the study. We randomly assigned 24 infant macaques between 3 and 7 days of age, when the first immunization was administered, to the various groups. For both vaccinations and sample collections, animals were anesthetized with 10 mg/kg of ketamine-HCl (Parke-Davis, Morris Plains, NJ) via the intramuscular (i.m.) route.

Animal immunization regimens. Based on the source of Env immunogens and adjuvants, the study was divided into two separate arms. In arm 1, the Env proteins gp120 C.1086 and gp120 TV1 plus the following adjuvants were provided by GSK: aluminum hydroxide [$Al(OH)_3$]; AS01, which is a liposome-based formulation containing MPL and QS-21 (*Quillaja saponaria* Molina fraction 21, licensed by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus, Inc., DE, USA); and MF59 (a trade name of Novartis). Env proteins and adjuvants were mixed according to the protocol provided by GSK. For the alum group, $Al(OH)_3$ was mixed with gp120 C.1086 and gp120 TV1 by magnetic agitation for 60 to 120 min. After addition of NaCl (1,500 mM), the Env protein-adjuvant mix was agitated for an additional 30 min. This Env protein-alum vaccine was allowed to mature for 7 days at 4°C prior to use for immunization. The Env vaccines adjuvanted with AS01 or MF59 were prepared on the day of immunization by mixing and agitating the two Env proteins and the adjuvant for 15 min. Animals were immunized immediately thereafter.

For the immunizations of the animals in arm 2, the recombinant gp120 proteins for C.1086_D7gp120K160N and TV1 were produced in 293T cells as described previously (46). Alum [$Al(OH)_3$] was obtained from the Vaccine Production Program at the Vaccine Research Center, National Institutes of Health. The GLA-SE and 3M-052-SE adjuvant formulations were prepared by IDRI. Env proteins and adjuvant were mixed just prior to immunization.

The animals in all groups were immunized at weeks 0, 2, and 6 by the i.m. route with 15 μg of C.1086 gp120 and 15 μg of TV1 gp120 plus adjuvant (Table 1).

Sample collection and processing. Samples for antibody analysis were collected at week 4 and 10, 2 weeks after the second and third immunizations, respectively. A single peripheral lymph node biopsy (axillary LN) was performed at week 10. All animals were followed for a total of 12 weeks, when a final blood sample was collected. Plasma was obtained from blood by centrifugation and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) and lymph node cell suspensions were prepared as described previously (29, 31).

Measurement of HIV Env-specific antibodies by ELISA. The plasma concentrations of HIV Env-specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (19). The concentration of HIV Env-specific IgG was calculated using a five-parameter fit curve relative to the standard using SoftMax Pro, version 6.3, software (Molecular Devices). To account for nonspecific binding, the positivity cutoff was selected as the concentration corresponding to three times the optical density (OD) of blank wells.

A binding antibody multiplex assay for plasma antibodies was performed as described previously (19). IgG binding was expressed as mean fluorescence intensity (MFI). To control for nonspecific binding, the MFI of sample binding to unconjugated blank beads was subtracted from the MFI of each antigen. An HIV-specific antibody response was considered positive if it had MFI values both above (i) the mean MFI plus 3 standard deviations (SD) of preimmunized plasma samples at week 0 and (ii) the lower detection limit of 100 MFI. The 50% effective concentration (EC_{50}) and maximum MFI values of the standards (b12R1, HIV IgG [HIVIG], and IgG from HIV-vaccinated rhesus macaques [RIVIG]) were tracked by Levy-Jennings charts to ensure consistency between assays (19).

Plasma antibody linear epitope mapping. Plasma IgG linear epitope mapping by peptide microarray was performed as previously described (19). Array slides were scanned at a wavelength of 635 nm with an InnoScan 710 scanner (Innopsys, Carbonne, France) using the extended dynamic range (XDR) mode. Images were analyzed using MagPix, version 7.4.1, software (Luminex Corporation) to obtain binding intensity values for all peptides. Binding values for preimmune serum from each animal were subtracted from the postimmunization binding values for each peptide. Binding magnitude to each epitope was defined as the highest binding to a single peptide within the epitope region.

Avidity assays. The avidity of anti-C.1086 gp120 or anti-TV1 gp120 plasma IgG antibodies was measured by surface plasmon resonance (SPR) as described previously (19). SPR measurements of purified plasma IgG avidity were performed using a Biacore 4000 instrument (GE Healthcare). Nonspecific binding of a preimmune (time zero) sample was subtracted from binding data for each postimmunization IgG sample. Data analyses were performed with BIAevaluation 4000 and BIAevaluation 4.1 software (BIAcore/GE Healthcare). Binding responses were measured by averaging postinjection response units (RU) over a 10-s window, and the dissociation rate constant, k_d (s^{-1}), was measured during postinjection phase (after stabilization of signal). Positive response was defined as when the RU value was ≥10. The relative avidity binding score is calculated as follows: avidity score (in response units) = (number of binding response units/k_d).

Neutralization assays. Neutralization of tier 1 and tier 2 clade C viruses by plasma was measured in TZM-bl cells by the reduction in luciferase reporter gene expression after a single round of infection, as described previously (47). Neutralization was assessed using MW965.LucR.T2A.ecto/293T (tier 1). The broadly neutralizing antibodies b12R1 and VRC01 were used as positive controls. The 50% inhibitory dose (ID_{50}) was calculated as the plasma dilution or antibody concentration that caused a 50% reduction

in number of relative light units (RLU) compared to values for the virus control wells after subtraction of the value for the controls. The preimmunization time point (week 0) was used to measure background levels of neutralization.

ADCC assay. The GranToxiLux (GTL) assay was used to detect plasma ADCC activity directed against $CCR5^+$ CEM.NKR T cells (AIDS Reagent Program, contributed by Alexandra Trkola) coated with C.1086 gp120 or clade C TV1 gp120 as described previously (19, 48). ADCC activity was measured in 4-fold serial plasma dilutions starting at 1:100. Cryopreserved human PBMCs from an HIV-seronegative donor with the 158F/V genotype for FcγRIIIa were used as the source of effector cells (49). Data were reported as the proportion of cells positive for proteolytically active granzyme B out of the total viable target cell population after subtraction of the background activity observed in wells containing effector and target cells in the absence of plasma. ADCC endpoint titers were determined by interpolating the dilutions of plasma that intercept the positive cutoff.

Detection of HIV Env-specific B cells. Lymph node cell suspensions were treated with 5 μM Chk2 Inhibitor II (Sigma) and 1% bovine serum albumin (BSA; Sigma) and blocked with 6.25 μg/ml anti-human CD4 (BD) at 4°C for 15 min. Cells were then costained with custom-conjugated BV421-gp120 (C.1086) and Alexa Fluor 647 (AF647)-gp120 (C.1086) prepared as described previously (50). Paraformaldehyde-fixed samples were acquired on a BD LSRFortessa instrument and analyzed using FlowJo software, version 10 (TreeStar, Ashland, OR). The following gating strategy was applied: lymphocytes were gated on singlets, live cells were selected to gate on $CD3^-$ T cells, $CD14^-$ (monocytes/macrophages) cells, or $CD20^+$ B cells. Only B cells positive for both BV421-gp120 and AF647-gp120 were considered HIV Env specific, and these populations were normalized against data from nonimmunized control animals.

Statistical analysis. Due to the pilot study nature of this research, the statistical analysis was limited. Results obtained in animals that received alum-adjuvanted Env protein could not be compared to results of any other vaccine group because a group size of 2 is too small. Vaccine responses were compared only between the AS01 and MF59 groups in arm 1 and between the 3M-052-SE and GLA-SE groups in arm 2. Data were analyzed by a Mann-Whitney test using GraphPad Prism Software (version 6; GraphPad, Inc., La Jolla, CA), with P value of <0.05 being considered statistically significant.

ACKNOWLEDGMENTS

We thank Jennifer Watanabe, Jodie Usachenko, Research Colony Services, and the Pathology, Veterinary, and Clinical Laboratory staff of the California National Primate Research Center for expert technical assistance. We thank Carolyn Weinbaum for her assistance with study coordination and Lawrence C. Armand, Neelima Choudhary, Thaddeus C. Gurley, Caroline L. Jones, Ryan Tuck, Tori Tuffman, R. Whitney Edwards, and Bob Wilson for expert technical assistance.

The work was supported by grant P01 AI117915 from the NIAID. In addition, this project was supported by a grant from the Office of Research Infrastructure Programs/OD (P51OD011107) to CNPRC. The UNC Flow Cytometry Core Facility is supported in part by a Cancer Center Core Support grant (P30 CA016086) to the UNC Lineberger Comprehensive Cancer Center. Research reported in this publication was supported by Center for AIDS Research award number 5P30AI050410.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Sallie Permar and Kristina De Paris designed the study; Koen K. A. Van Rompay was responsible for all animal procedures; Bonnie Phillips managed the study procedures and data analysis; Jennifer Rodriguez-Nieves processed samples; Genevieve Fouda, Josh Eudailey, Maria Dennis, and Xiaoying Shen performed the antibody analysis under the leadership of Sallie Permar and Anthony Moody; Justin Pollara and Guido Ferrari performed ADCC assays; avidity was assessed by S. Munir Alam; the statistical analysis was performed by Michael Hudgens; Clarisse Lorin, Marguerite Koutsoukos, Mark Tomai, and Christopher B. Fox provided the adjuvants and input in experimental design; the manuscript was written by Kristina De Paris with input from all coauthors.

C.L. and M.K. are employees of the GSK group of companies and report owning GSK shares and/or restricted GSK shares.

REFERENCES

1. Crotty S. 2015. A brief history of T cell help to B cells. Nat Rev Immunol 15:185–189. https://doi.org/10.1038/nri3803.
2. Levy O, Suter EE, Miller RL, Wessels MR. 2006. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood 108:1284–1290. https://doi.org/10.1182/blood-2005-12-4821.
3. Xu H, Wang X, Lackner AA, Veazey RS. 2014. PD-1^{HIGH} follicular CD4 T helper cell subsets residing in lymph node germinal centers correlate with B cell maturation and IgG production in rhesus macaques. Front Immunol 5:85. https://doi.org/10.3389/fimmu.2014.00085.
4. Mastelic B, Kamath AT, Fontannaz P, Tougne C, Rochat AF, Belnoue E, Combescure C, Auderset F, Lambert PH, Tacchini-Cottier F, Siegrist CA. 2012. Environmental and T cell-intrinsic factors limit the expansion of

neonatal follicular T helper cells but may be circumvented by specific adjuvants. J Immunol 189:5764–5772. https://doi.org/10.4049/jimmunol.1201143.
5. Ridings J, Nicholson IC, Goldsworthy W, Haslam R, Roberton DM, Zola H. 1997. Somatic hypermutation of immunoglobulin genes in human neonates. Clin Exp Immunol 108:366–374. https://doi.org/10.1046/j.1365-2249.1997.3631264.x.
6. Knudsen NP, Olsen A, Buonsanti C, Follmann F, Zhang Y, Coler RN, Fox CB, Meinke A, D'Oro U, Casini D, Bonci A, Billeskov R, De Gregorio E, Rappuoli R, Harandi AM, Andersen P, Agger EM. 2016. Different human vaccine adjuvants promote distinct antigen-independent immunological signatures tailored to different pathogens. Sci Rep 6:19570. https://doi.org/10.1038/srep19570.
7. Garcon N, Di Pasquale A. 2017. From discovery to licensure, the adjuvant system story. Hum Vaccin Immunother 13:19–33. https://doi.org/10.1080/21645515.2016.1225635.
8. Dowling DJ, Levy O. 2014. Ontogeny of early life immunity. Trends Immunol 35:299–310. https://doi.org/10.1016/j.it.2014.04.007.
9. Philbin VJ, Dowling DJ, Gallington LC, Cortes G, Tan Z, Suter EE, Chi KW, Shuckett A, Stoler-Barak L, Tomai M, Miller RL, Mansfield K, Levy O. 2012. Imidazoquinoline Toll-like receptor 8 agonists activate human newborn monocytes and dendritic cells through adenosine-refractory and caspase-1-dependent pathways. J Allergy Clin Immunol 130:195–204 e9. https://doi.org/10.1016/j.jaci.2012.02.042.
10. Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, Mshamu S, Lang T, Gould J, Dubois MC, Demoitie MA, Stallaert JF, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S, Mturi N, Drakeley CJ, Savarese B, Villafana T, Ballou WR, Cohen J, Riley EM, Lemnge MM, Marsh K, von Seidlein L. 2008. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 359:2521–2532. https://doi.org/10.1056/NEJMoa0807381.
11. Weinberger B. 2018. Adjuvant strategies to improve vaccination of the elderly population. Curr Opin Pharmacol 41:34–41. https://doi.org/10.1016/j.coph.2018.03.014.
12. Weinberger B. 2018. Vaccines for the elderly: current use and future challenges. Immun Ageing 15:3. https://doi.org/10.1186/s12979-017-0107-2.
13. Levy O, Zarember KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. 2004. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol 173:4627–4634. https://doi.org/10.4049/jimmunol.173.7.4627.
14. Vasilakos JP, Tomai MA. 2013. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev Vaccines 12:809–819. https://doi.org/10.1586/14760584.2013.811208.
15. Dowling DJ, van Haren SD, Schneid A, Bergelson I, Kim D, Mancuso CJ, Foppen W, Ozonoff A, Fresh L, Theriot TB, Lackner AA, Ficherova RN, Smirnov D, Vasilakos JP, Beaurline JM, Tomai MA, Midkiff CC, Alvarez X, Blanchard JL, Gilbert MH, Aye PP, Levy O. 2017. TLR7/8 adjuvant overcomes newborn hyporesponsiveness to pneumococcal conjugate vaccine at birth. JCI Insight 2:e91020. https://doi.org/10.1172/jci.insight.91020.
16. Leroux-Roels G, Maes C, Willekens J, De Boever F, de Rooij R, Martell L, Bedell L, Wittke F, Slobod K, Dull P. 2016. A randomized, observer-blind phase Ib study to identify formulations and vaccine schedules of a trivalent group B *Streptococcus* vaccine for use in non-pregnant and pregnant women. Vaccine 34:1786–1791. https://doi.org/10.1016/j.vaccine.2016.02.044.
17. Behzad H, Huckriede AL, Haynes L, Gentleman B, Coyle K, Wilschut JC, Kollmann TR, Reed SG, McElhaney JE. 2012. GLA-SE, a synthetic Toll-like receptor 4 agonist, enhances T-cell responses to influenza vaccine in older adults. J Infect Dis 205:466–473. https://doi.org/10.1093/infdis/jir769.
18. Heeke DS, Lin R, Rao E, Woo JC, McCarthy MP, Marshall JD. 2016. Identification of GLA/SE as an effective adjuvant for the induction of robust humoral and cell-mediated immune responses to EBV-gp350 in mice and rabbits. Vaccine 34:2562–2569. https://doi.org/10.1016/j.vaccine.2016.04.012.
19. Phillips B, Fouda GG, Eudailey J, Pollara J, Curtis AD, II, Kunz E, Dennis M, Shen X, Bay C, Hudgens M, Pickup D, Alam SM, Ardeshir A, Kozlowski PA, Van Rompay KKA, Ferrari G, Moody MA, Permar S, De Paris K. 2017. Impact of poxvirus vector priming, protein coadministration, and vaccine intervals on HIV gp120 vaccine-elicited antibody magnitude and function in infant macaques. Clin Vaccine Immunol 24:e00231-17. https://doi.org/10.1128/CVI.00231-17.
20. Zambonelli C, Dey AK, Hilt S, Stephenson S, Go EP, Clark DF, Wininger M, Labranche C, Montefiori D, Liao HX, Swanstrom RI, Desaire H, Haynes BF, Carfi A, Barnett SW. 2016. Generation and characterization of a bivalent HIV-1 subtype C gp120 protein boost for proof-of-concept HIV vaccine efficacy trials in southern Africa. PLoS One 11:e0157391. https://doi.org/10.1371/journal.pone.0157391.
21. Garcia-Arriaza J, Perdiguero B, Heeney J, Seaman M, Montefiori DC, Labranche C, Yates NL, Shen X, Tomaras GD, Ferrari G, Foulds KE, McDermott A, Kao SF, Roederer M, Hawkins N, Self S, Yao J, Farrell P, Phogat S, Tartaglia J, Barnett SW, Burke B, Cristillo A, Weiss D, Lee C, Kibler K, Jacobs B, Asbach B, Wagner R, Ding S, Pantaleo G, Esteban M. 2015. Head-to-head comparison of poxvirus NYVAC and ALVAC vectors expressing identical HIV-1 clade C immunogens in prime-boost combination with Env protein in nonhuman primates. J Virol 89:8525–8539. https://doi.org/10.1128/JVI.01265-15.
22. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH. 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 366:1275–1286. https://doi.org/10.1056/NEJMoa1113425.
23. Rerks-Ngarm S, Paris RM, Chunsutthiwat S, Premsri N, Namwat C, Bowonwatanuwong C, Li SS, Kaewkungkal J, Trichavaroj R, Churikanont N, de Souza MS, Andrews C, Francis D, Adams E, Flores J, Gurunathan S, Tartaglia J, O'Connell RJ, Eamsila C, Nitayaphan S, Ngauy V, Thongcharoen P, Kunasol P, Michael NL, Robb ML, Gilbert PB, Kim JH. 2013. Extended evaluation of the virologic, immunologic, and clinical course of volunteers who acquired HIV-1 infection in a phase III vaccine trial of ALVAC-HIV and AIDSVAX B/E. J Infect Dis 207:1195–1205. https://doi.org/10.1093/infdis/jis478.
24. Borkowsky W, Wara D, Fenton T, McNamara J, Kang M, Mofenson L, McFarland E, Cunningham C, Duliege AM, Francis D, Bryson Y, Burchett S, Spector SA, Frenkel LM, Starr S, Van Dyke R, Jimenez E. 2000. Lymphoproliferative responses to recombinant HIV-1 envelope antigens in neonates and infants receiving gp120 vaccines. AIDS Clinical Trial Group 230 Collaborators. J Infect Dis 181:890–896.
25. Cunningham CK, Wara DW, Kang M, Fenton T, Hawkins E, McNamara J, Mofenson L, Duliege AM, Francis D, McFarland EJ, Borkowsky W, Pediatric AIDS Clinical Trials Group 230 Collaborators. 2001. Safety of 2 recombinant human immunodeficiency virus type 1 (HIV-1) envelope vaccines in neonates born to HIV-1-infected women. Clin Infect Dis 32:801–807. https://doi.org/10.1086/319215.
26. McFarland EJ, Borkowsky W, Fenton T, Wara D, McNamara J, Samson P, Kang M, Mofenson L, Cunningham C, Duliege AM, Sinangil F, Spector SA, Jimenez E, Bryson Y, Burchett S, Frenkel LM, Yogev R, Gigliotti F, Luzuriaga K, Livingston RA, AIDS Clinical Trial Group 230 Collaborators. 2001. Human immunodeficiency virus type 1 (HIV-1) gp120-specific antibodies in neonates receiving an HIV-1 recombinant gp120 vaccine. J Infect Dis 184:1331–1335. https://doi.org/10.1086/323994.
27. Fouda GG, Cunningham CK, McFarland EJ, Borkowsky W, Muresan P, Pollara J, Song LY, Liebl BE, Whitaker K, Shen X, Vandergrift NA, Overman RG, Yates NL, Moody MA, Fry C, Kim JH, Michael NL, Robb M, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Liao HX, Haynes BF, Montefiori DC, Ferrari G, Tomaras GD, Permar SR. 2015. Infant HIV type 1 gp120 vaccination elicits robust and durable anti-V1V2 immunoglobulin G responses and only rare envelope-specific immunoglobulin A responses. J Infect Dis 211:508–517. https://doi.org/10.1093/infdis/jiu444.
28. Abel K. 2009. The rhesus macaque pediatric SIV infection model—a valuable tool in understanding infant HIV-1 pathogenesis and for designing pediatric HIV-1 prevention strategies. Curr HIV Res 7:2–11. https://doi.org/10.2174/157016209787048528.
29. Jensen K, Nabi R, Van Rompay KK, Robichaux S, Lifson JD, Piatak M, Jr, Jacobs WR, Jr, Fennelly G, Canfield D, Mollan KR, Hudgens MG, Larsen MH, Amedee AM, Kozlowski PA, De Paris K. 2016. Vaccine-elicited mucosal and systemic antibody responses are associated with reduced simian immunodeficiency viremia in infant rhesus macaques. J Virol 90:7285–7302. https://doi.org/10.1128/JVI.00481-16.
30. Jensen K, Pena MG, Wilson RL, Ranganathan UD, Jacobs WR, Jr, Fennelly G, Larsen M, Van Rompay KK, Kozlowski PA, Abel K. 2013. A neonatal oral *Mycobacterium tuberculosis*-SIV prime/intramuscular MVA-SIV boost combination vaccine induces both SIV and *Mtb*-specific immune re-

sponses in infant macaques. Trials Vaccinol 2:53–63. https://doi.org/10.1016/j.trivac.2013.09.005.
31. Marthas ML, Van Rompay KK, Abbott Z, Earl P, Buonocore-Buzzelli L, Moss B, Rose NF, Rose JK, Kozlowski PA, Abel K. 2011. Partial efficacy of a VSV-SIV/MVA-SIV vaccine regimen against oral SIV challenge in infant macaques. Vaccine 29:3124–3137. https://doi.org/10.1016/j.vaccine.2011.02.051.
32. Van Rompay KK, Abel K, Earl P, Kozlowski PA, Easlick J, Moore J, Buonocore-Buzzelli L, Schmidt KA, Wilson RL, Simon I, Moss B, Rose N, Rose J, Marthas ML. 2010. Immunogenicity of viral vector, prime-boost SIV vaccine regimens in infant rhesus macaques: attenuated vesicular stomatitis virus (VSV) and modified vaccinia Ankara (MVA) recombinant SIV vaccines compared to live-attenuated SIV. Vaccine 28:1481–1492. https://doi.org/10.1016/j.vaccine.2009.11.061.
33. Van Rompay KK, Abel K, Lawson JR, Singh RP, Schmidt KA, Evans T, Earl P, Harvey D, Franchini G, Tartaglia J, Montefiori D, Hattangadi S, Moss B, Marthas ML. 2005. Attenuated poxvirus-based simian immunodeficiency virus (SIV) vaccines given in infancy partially protect infant and juvenile macaques against repeated oral challenge with virulent SIV. J Acquir Immune Defic Syndr 38:124–134. https://doi.org/10.1097/00126334-200502010-00002.
34. Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, Gilbert PB, Huang Y, Gurley TC, Kozink DM, Marshall DJ, Whitesides JF, Tsao CY, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Kim JH, Michael NL, Tomaras GD, Montefiori DC, Lewis GK, DeVico A, Evans DT, Ferrari G, Liao HX, Haynes BF. 2012. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. J Virol 86:11521–11532. https://doi.org/10.1128/JVI.01023-12.
35. Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J, Bonsignori M, Moody MA, Fong Y, Chen X, Poling B, Nicholson CO, Zhang R, Lu X, Parks R, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Gilbert PB, Kim JH, Michael NL, Montefiori DC, Haynes BF. 2013. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proc Natl Acad Sci U S A 110:9019–9024. https://doi.org/10.1073/pnas.1301456110.
36. Gilbert P, Wang M, Wrin T, Petropoulos C, Gurwith M, Sinangil F, D'Souza P, Rodriguez-Chavez IR, DeCamp A, Giganti M, Berman PW, Self SG, Montefiori DC. 2010. Magnitude and breadth of a nonprotective neutralizing antibody response in an efficacy trial of a candidate HIV-1 gp120 vaccine. J Infect Dis 202:595–605. https://doi.org/10.1086/654816.
37. Karnasuta C, Akapirat S, Madnote S, Savadsuk H, Puangkaew J, Rittiroongrad S, Rerks-Ngarm S, Nitayaphan S, Pitisuttithum P, Kaewkungwal J, Tartaglia J, Sinangil F, Francis DP, Robb ML, de Souza MS, Michael NL, Excler JL, Kim JH, O'Connell RJ, Karasavvas N. 2017. Comparison of antibody responses induced by RV144, VAX003, and VAX004 vaccination regimens. AIDS Res Hum Retroviruses 33:410–423. https://doi.org/10.1089/aid.2016.0204.
38. Holbrook BC, D'Agostino RB, Jr, Tyler Aycock S, Jorgensen MJ, Hadimani MB, Bruce King S, Alexander-Miller MA. 2017. Adjuvanting an inactivated influenza vaccine with conjugated R848 improves the level of antibody present at 6 months in a nonhuman primate neonate model. Vaccine 35:6137–6142. https://doi.org/10.1016/j.vaccine.2017.09.054.
39. Holbrook BC, Aycock ST, Machiele E, Clemens E, Gries D, Jorgensen MJ, Hadimani MB, King SB, Alexander-Miller MA. 2018. An R848 adjuvanted influenza vaccine promotes early activation of B cells in the draining lymph nodes of non-human primate neonates. Immunology 153:357–367. https://doi.org/10.1111/imm.12845.
40. Reis e Sousa C. 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. Semin Immunol 16:27–34. https://doi.org/10.1016/j.smim.2003.10.004.
41. Kawasaki T, Kawai T. 2014. Toll-like receptor signaling pathways. Front Immunol 5:461. https://doi.org/10.3389/fimmu.2014.00461.
42. Colak E, Leslie A, Zausmer K, Khatamzas E, Kubarenko AV, Pichulik T, Klimosch SN, Mayer A, Siggs O, Hector A, Fischer R, Klesser B, Rautanen A, Frank M, Hill AV, Manoury B, Beutler B, Hartl D, Simmons A, Weber AN. 2014. RNA and imidazoquinolines are sensed by distinct TLR7/8 ectodomain sites resulting in functionally disparate signaling events. J Immunol 192:5963–5973. https://doi.org/10.4049/jimmunol.1303058.
43. Barton GM, Kagan JC. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol 9:535–542. https://doi.org/10.1038/nri2587.
44. National Research Council. 2011. Guide for the care and use of laboratory animals, 8th ed. The National Academies Press, Washington, DC.
45. Council for International Organizations of Medical Sciences. 2012. International guiding principles for biomedical research involving animals. Council for International Organizations of Medical Sciences, Geneva, Switzerland. https://olaw.nih.gov/sites/default/files/Guiding_Principles_2012.pdf.
46. Fouda GG, Amos JD, Wilks AB, Pollara J, Ray CA, Chand A, Kunz EL, Liebl BE, Whitaker K, Carville A, Smith S, Colvin L, Pickup DJ, Staats HF, Overman G, Eutsey-Lloyd K, Parks R, Chen H, Labranche C, Barnett S, Tomaras GD, Ferrari G, Montefiori DC, Liao HX, Letvin NL, Haynes BF, Permar SR. 2013. Mucosal immunization of lactating female rhesus monkeys with a transmitted/founder HIV-1 envelope induces strong Env-specific IgA antibody responses in breast milk. J Virol 87:6986–6999. https://doi.org/10.1128/JVI.00528-13.
47. Montefiori DC. 2009. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol 485:395–405. https://doi.org/10.1007/978-1-59745-170-3_26.
48. Pollara J, Hart L, Brewer F, Pickeral J, Packard BZ, Hoxie JA, Komoriya A, Ochsenbauer C, Kappes JC, Roederer M, Huang Y, Weinhold KJ, Tomaras GD, Haynes BF, Montefiori DC, Ferrari G. 2011. High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. Cytometry A 79:603–612. https://doi.org/10.1002/cyto.a.21084.
49. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. 1997. FcγRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell FcγRIIIa, independently of the FcγRIIIa-48L/R/H phenotype. Blood 90:1109–1114.
50. Moody MA, Yates NL, Amos JD, Drinker MS, Eudailey JA, Gurley TC, Marshall DJ, Whitesides JF, Chen X, Foulger A, Yu JS, Zhang R, Meyerhoff RR, Parks R, Scull JC, Wang L, Vandergrift NA, Pickeral J, Pollara J, Kelsoe G, Alam SM, Ferrari G, Montefiori DC, Voss G, Liao HX, Tomaras GD, Haynes BF. 2012. HIV-1 gp120 vaccine induces affinity maturation in both new and persistent antibody clonal lineages. J Virol 86:7496–7507. https://doi.org/10.1128/JVI.00426-12.